EOTAXIN : EOSINOPHIL CHEMOTACTIC CYTOKINE

The present invention relates to a chemotactic cytokine.

5 The accumulation of eosinophil leukocytes is a characteristic
feature of IgE-mediated allergic reactions such as allergic
asthma, rhinitis and eczema. Eosinophil accumulation also
occurs in non-allergic asthma. The immediate broncho-
constriction in response to a provoking stimulus in the
10 asthmatic involves mast cell activation and the release of
constrictor mediators. This is followed after several hours
in some individuals by a late bronchoconstrictor response
associated with a massive influx of eosinophils (1). Repeated
provocation results in chronic inflammation in the airways and
15 a marked hyper-responsiveness to constrictor mediators. The
magnitude of both the late response and the chronic hyper-
responsiveness correlates with the numbers of eosinophils
present in the lung (2,3).

20 The present invention provides a chemoattractant protein
capable of attracting eosinophils and of inducing eosinophil
accumulation and/or activation _in vitro_ and _in vivo_. The
chemoattractant protein of the present invention is designated
"eotaxin".
25
Eotaxins are proteins of the C-C branch of the platelet factor
4 superfamily of chemotactic cytokines. Within the C-C branch
of the platelet factor 4 superfamily of chemotactic cytokines,
or chemokines, certain members have the property of attracting
30 eosinophils _in vitro_ and some may induce eosinophil
accumulation _in vivo_. For example, the chemokines RANTES and
MIP-1α attract eosinophils _in vitro_ while MCP-1 and MIP-1ß do
not. ("RANTES" denotes Regulated upon Activation in Normal T
cells Expressed and Secreted, "MIP" denotes Macrophage
35 Inflammatory Protein, and "MCP" denotes Monocyte Chemo-
attractant Protein.)

Naturally-occurring cytokines within the platelet factor 4
superfamily of chemotactic cytokines may have marked inter-

the amino acid sequence of the protein, and in the
carbohydrate modifications of the protein, while retaining the
same characteristic functional properties. Similar variations
in structure may occur in cytokines obtained from different
5 individuals within the same species. Many chemokines within
the C-C branch of the platelet factor 4 superfamily show
promiscuity of receptor binding, and the ability of different
chemokines to bind to the same receptor is not necessarily
dependent on a high degree of homology at the amino acid
10 level. Accordingly, both interspecies and intraspecies
variations in protein length, amino acid sequence and
carbohydrate modifications are generally to be expected for
eotaxins.

15 The ability to attract eosinophils and to induce eosinophil
accumulation and/or activation _in vitro_ and _in vivo_ is a
characteristic property of eotaxins. Furthermore, eotaxins
generally show substantially no attractive effect for neutro-
phils _in vivo_. The eosinophil chemoattractant effect may be
20 an inter-species effect, for example, guinea-pig eotaxin
appears to be potent in inducing chemotaxis of human eosino-
phils in vitro.

An eotaxin may be obtained from an appropriate body fluid, for
25 example, from bronchoalveolar lavage fluid obtained from a
human or non-human subject, particularly an allergic subject
after an allergen challenge, either experimentally induced or
naturally incurred. Other sources of eotaxins are, for
example, inflammatory exudate fluids and _in vitro_ cultures of
30 macrophages, lymphocytes, neutrophils, mast cells, airway
epithelial cells, connective tissue cells, vascular
endothelial cells and eosinophils themselves.

For example, an eotaxin may be obtained from a sensitised
35 guinea-pig after allergen challenge. Guinea-pig models are
useful as they share many common features with the asthmatic
response in man. Eotaxin obtainable from bronchoalveolar

lavage fluid of a sensitised guinea-pig by sequential HPLC
purification generally has a molecular weight in the range of
from 6-16 kDa. (As indicated above, intraspecies molecular
weight variations of this order of magnitude are observed in
5 members of the platelet factor 4 superfamily.)

The amino acid sequence of a guinea-pig eotaxin is set out in
SEQ.ID. NO. 1, SEQ.ID. NO. 2 and in Figures 7 and 8 of the
accompanying drawings. Other guinea-pig eotaxins will
10 generally have at least 50% overall homology with the sequence
shown in SEQ.ID. NO. 1 (Figure 7) at the amino acid level. The
homology may be at least 60%, for example at least 70%, for
example at least 80% with the sequence set out in SEQ.ID. NO.
1 and in Figure 7.
15

Percentage homology in the present case is calculated on the
basis of amino acids that are identical in corresponding
positions in the two sequences under investigation.
Conservative substitutions are not taken into account. In the
20 calculation of percentage homology of a putative eotaxin
molecule under investigation with the sequence shown in
SEQ.ID. NO. 1 (Figure 7) or with SEQ.ID. NO. 2 (Figure 8) if
the molecule under investigation has a different length from
the eotaxin set out in SEQ.ID. NO. 1 or SEQ.ID. NO. 2 (Figure
25 7 or Figure 8), then the calculation is based on the amino
acids in the portion of the molecule under investigation that
overlaps with the sequence shown in SEQ.ID. NO. 1 (Figure 7)
or SEQ.ID. NO. 2 (Figure 8). Software packages for the
alignment of amino acid sequences and the calculation of
30 homology are available commercially, for example, the
"Bestfit" program available from Genetics Computer Group
Sequence Analysis Software, Madison, Wisconsin, U.S.A..

Unless specified otherwise, the specific values of percentage
35 homology between eotaxin and other chemotactic cytokines given
in the present specification have been calculated on the basis
of the eotaxin set out in SEQ.ID. NO. 1 (Figure 7).

As indicated above, eotaxins obtainable from species other
than guinea-pigs, for example humans, will exhibit inter-
species differences of the type demonstrated by other members
of the C-C branch of the platelet factor 4 superfamily of
5 chemokines, for example, differences in protein length, amino
acid sequence and carbohydrate modifications. There may, for
example, be variations in the C- and/or N-terminal residues.
For example, it is expected that the molecular weight of an
eotaxin from a species other than guinea-pig will generally
10 fall within the range of from 6 kDa to 16 kDa, but in some
cases an eotaxin may have a molecular weight less than 6 kDa
or more than 16 kDa.

Similarly, it is expected that in general an eotaxin from a
15 species other than guinea-pig will have at least 40% overall
homology with the sequence set out in SEQ.ID. NO. 1 and in
Figure 7. The homology may be at least 50%, for example at
least 60%, for example at least 70%, for example at least 80%
with the sequence set out in SEQ.ID. NO. 1 and in Figure 7.
20 There may, however, be eotaxins from species other than
guinea-pigs that have less than 40% homology with SEQ.ID. NO.
1 (Figure 7).

Eotaxins may be identified by any one or more of the
25 characteristics set out above, in particular by their ability
to attract and/or active eosinophils in vitro and cause their
accumulation and/or activation in vivo. A characteristic that
assists the identification of a molecule as an eotaxin is the
lack of attractive effect on neutrophils.
30
The present invention provides a method of determining the
ability of a substance to induce eosinophil accumulation
and/or activation in vivo, that is to say, a method for
testing putative eotaxins, which comprises administering the
35 substance, generally intradermally, to a test animal
previously treated with labelled, for example ^{111}In-labelled,
eosinophils and subsequently determining the number of

labelled eosinophils at a skin site.

One in vitro method that may be used to test a putative
eotaxin for the ability to attract and/or activate eosinophils
5 in vitro is the ability of the substance to increase
eosinophil intracellular calcium levels. Other general
methods for determining chemotactic activity in vitro may be
used to test putative eotaxins in vitro.

10 Confirmation that an eosinophil attractant is an eotaxin may
also be made by consideration of sequence homology of that
protein with the sequence set out in SEQ.ID. NO. 1 (Figure 7)
and/or with the sequence set in SEQ.ID. NO. 2 (Figure 8)
and/or by consideration of the structural relationship between
15 the protein and the guinea-pig eotaxin.

As mentioned above, RANTES and MIP-1α are both eosinophil
activators. Eotaxin has functional similarities but low
structural homology with RANTES and MIP-1α (31% homology with
20 MIP-1α at the amino acid level calculated on the basis of
SEQ.ID. No. 1 (Figure 7); 32% homology when calculated on the
basis of the overlapping sequences and 26% homology with
RANTES at the amino acid level calculated on the basis of
SEQ.ID. No. 1 (Figure 7); 27% homology when calculated on the
25 basis of the overlapping sequences). An eotaxin can be
distinguished from RANTES and MIP-1α not only by the degree of
homology but also by the overall differences in sequence and
structure.

30 In addition to full-length eotaxin molecules, the present
invention also provides molecules that comprise less than a
full length eotaxin sequence. Such molecules (called
"fragments" herein) may be polypeptides or peptides. For use
as an eotaxin substitute, a fragment should retain one or
35 more of the biological activities of the parent molecule.

Eosinophils contain an armoury of chemicals necessary for

killing parasites. These chemicals have been implicated in
the damage to airway epithelium that occurs in asthma and may
relate to the observed changes in airway function (26,27).
From our studies we suggest that eotaxins should be considered
5 as important mediators of eosinophil accumulation in vivo.
Macrophages, lymphocytes, neutrophils, mast cells, airway
epithelial cells, connective tissue cells, vascular
endothelial cells and eosinophils themselves are likely
candidates as the source of the eosinophil chemoattractant
10 activity generated in the lung. Platelets may also have a
role as it has been shown that they can release C-C chemokines
(22). Further, an early platelet deposition may be involved
in the subsequent eosinophil accumulation in vivo (28,29) and
there is evidence that platelet-activating factor induces the
15 synthesis of an unidentified eosinophil chemoattractant in
vivo (30). In this respect, it is of interest that platelet-
derived growth factor can induce gene expression of C-C
chemokines in fibroblasts (31). Furthermore, the C-C chemo-
kines have been implicated in wound healing (18). This may be
20 important in the sub-epithelial basement membrane fibrosis
that is a prominent feature of the asthmatic lung. Thus,
eotaxins may be involved in both eosinophil accumulation and
in chronic structural changes in the lung.

25 Eotaxins may have an important role in asthma and in other
diseases having an inflammatory component where eosinophil
accumulation and/or activation is a prominent feature, for
example, rhinitis and eczema, especially allergic eczema.
Accordingly, agents that inhibit or otherwise hinder the
30 production, release or action of eotaxins have potential as
selective therapeutic agents. Such agents and their
therapeutic use are part of the present invention.

Such agents include inhibitors that affect the interaction of
35 an eotaxin with eotaxin receptors, for example, by binding to
an eotaxin or to an eotaxin receptor. An example of such an
inhibitor is receptors themselves which, on administration,

can bind an eotaxin and prevent its interaction with
naturally-occurring receptors. Such inhibitory receptors may
be soluble or insoluble. Receptors which are not involved in
cell activation may be bound to, or induced on, cells. Such
5 receptors may also be used to remove endogenous eotaxin.

Further examples of agents that affect the interaction of
eotaxins with eotaxin receptors are receptor antagonists, and
antibodies, both antibodies directed against (capable of
10 binding with) an eotaxin and antibodies directed against an
eotaxin receptor, especially monoclonal antibodies. Any other
agent that inhibits or otherwise hinders the binding of an
eotaxin to an eotaxin receptor also has therapeutic potential,
for example, any other agent that binds to an eotaxin or to an
15 eotaxin receptor. Further agents that have therapeutic
potential are those that prevent or reduce activation of
eotaxin receptors.

Further agents that inhibit or otherwise hinder the action of
20 eotaxins are those that change the structure of an eotaxin
such that it is no longer able to bind to an eotaxin receptor,
for example, an enzyme or other agent that degrades eotaxin
specifically.

25 Receptor promiscuity is common among chemokines, so although
it is essential that a receptor is capable of binding an
eotaxin, the receptor need not necessarily be eotaxin-
specific. For example, a receptor may bind MIP-1α, RANTES
and/or other eosinophil attractant chemokines as well as an
30 eotaxin.

As indicated above, possibilities for therapeutic intervention
include the use of a receptor to which an eotaxin binds,
especially a soluble receptor. It may be advantageous to use
35 an eotaxin-specific receptor. Further possibilities for
therapeutic intervention include receptor antagonists, for
example, based on 3-dimensional structures or the amino acid

sequences of eotaxins and/or of eotaxin receptors, and agents
found to inhibit eotaxin or other agonists binding to or
activating eotaxin receptors. For example, a receptor
antagonist or an agonist inhibitor may be a polypeptide in
5 which the sequence of a full-length naturally-occurring
eotaxin has been modified, for example, by amino acid
substitution, or may be a fragment of an eotaxin (that is to
say, a polypeptide or small peptide comprising part of the
amino acid sequence of a naturally-occurring eotaxin), or a
10 modified fragment of an eotaxin, for example, modified by
amino acid substitution.

Furthermore, knowledge of the sequence and/or structure of
eotaxins either alone or in combination with knowledge of the
15 sequence and/or structure of other chemokines that bind to the
same receptor(s) as eotaxins, provides useful information for
the design of therapeutic agents.

Agents that prevent or inhibit eotaxin synthesis or release
20 may also be used therapeutically. Such agents and their use
are also part of the present invention.

All inhibitors of eotaxin activity, synthesis and release,
including soluble receptors, antibodies, antagonists and
25 inhibitors of agonist binding, and their use are part of the
present invention.

The present invention accordingly provides an agent that
inhibits or otherwise hinders the production, release or
30 action of an eotaxin, especially an agent as described above,
for use as a medicament. The invention also provides the use
of an agent that inhibits or otherwise hinders the production,
release or action of an eotaxin, especially an agent as
described above, in the manufacture of a medicament for the
35 treatment of asthma or another disease having an inflammatory
component, particularly with accumulation of eosinophils, for
example, rhinitis or eczema, especially allergic eczema.

The use of the structural and sequence information relating to
eotaxins in the design of therapeutically and diagnostically
useful agents, for example, in computer-aided design based on
the three dimensional structure of eotaxins is part of the
5 present invention.

Putative inhibitors of eotaxin activity may be screened using
in vivo and in vitro assays based on inhibition of
chemoattraction and/or accumulation and/or activation of
10 eosinophils by eotaxins. Some general methods for testing the
activity of a compound for an inhibitory effect on the
activity of a chemoattractant cytokine in vitro are known.
Such assays may be used to determine the inhibitory action of
a putative inhibitor on in vitro effects induced in eosino-
15 phils by eotaxins.

Assays that are suitable for screening putative eotaxin
inhibitors include, for example, inhibition in vitro of
elevation of intracellular calcium levels induced in cells by
20 eotaxin. The method of the present invention for determining
the ability of a substance to induce eosinophil accumulation
and/or activation in vivo, that is to say, a method for
testing putative eotaxins, may also be used to determine the
ability of a substance to inhibit eosinophil accumulation
25 and/or activation induced in vivo by an eotaxin: an animal is
pretreated with labelled eosinophils, an eotaxin and a
putative inhibitor are administered, and the number of
labelled eosinophils at a skin site are determined
subsequently. The eotaxin is generally administered intra-
30 dermally, and the putative inhibitor may be administered by
the same route or by a different route, for example
systemically.

Examples of in vitro and in vivo assays both for the
35 determination of eotaxin activity and for the determination of
eotaxin inhibitory activity are described herein. For
example, Example 1 gives a detailed protocol for the in vivo

assay of the present invention, and Example 4 gives detailed
protocols of various assays. The assays described herein may
be used as such, or may be modified as required. Assays may
be used alone or in combination to establish eotaxin and
5 eotaxin-inhibitory activity. A putative inhibitors may be
any of the types of molecules described above, including
receptors, for example, soluble receptors, antibodies, and
antagonists and inhibitors of agonist binding. Methods for
testing putative inhibitors of eotaxins are also part of the
10 present invention.

A further aspect of the present invention is a pharmaceutical
preparation comprising, as active ingredient, an agent that
inhibits or otherwise hinders the production, release or
15 action of an eotaxin, in admixture or in conjunction with a
pharmaceutically suitable carrier. Such agents are described
above and include, for example, an inhibitor of eotaxin
synthesis or release, a soluble eotaxin receptor, an eotaxin
receptor antagonist or an inhibitor of an eotaxin receptor
20 agonist, an antibody against eotaxin or an antibody against an
eotaxin receptor.

The invention further provides a method of treating asthma and
other inflammatory diseases, comprising the administration of
25 an effective amount of an agent that inhibits or otherwise
hinders the production, release or action of an eotaxin. The
agent may be as described above, for example, an inhibitor of
eotaxin synthesis or release, a soluble eotaxin receptor, an
eotaxin receptor antagonist or an inhibitor of an eotaxin
30 receptor agonist, or an antibody against eotaxin or against an
eotaxin receptor.

The present invention also provides assays for eotaxins and
for anti-eotaxin antibodies, especially immunoassays and in
35 particular ELISAs (enzyme-linked immunosorbent assays). The
invention provides, for example, an immunoassay for an
antigen, characterised in that the antigen is an eotaxin, and

also provides an immunoassay for an antibody, characterised in
that the antibody is an anti-eotaxin antibody. The invention
also provides assays for eotaxins that are analogous to
immunoassays for eotaxins but that use a specific-binding
5 partner other than an antibody. In such specific-binding
partner assays an eotaxin receptor may be used instead of an
anti-eotaxin antibody.

In an immunoassay, an anti-eotaxin antibody may, for example,
10 be coated on a solid surface to enable capture and hence
detection of eotaxin. An anti-eotaxin antibody may be used in
an assay for the detection of antibodies to eotaxin, for
example, in a competitive antibody assay. A labelled eotaxin
or a derivative thereof, for example, a recombinant eotaxin or
15 a synthetic peptide comprising part of the amino acid sequence
of an eotaxin may be used in a competitive antigen assay for
eotaxin or may be used to coat a solid surface in a capture
assay for antibodies to eotaxin. The many different types of
assay format are well described in the literature of the art,
20 see for example "ELISA and Other Solid Phase Immunoassays,
Theoretical and Practical Aspects" Eds. Kemeny D.M. &
Challacombe S.J., John Wiley, 1988. (36). Assays using an
eotaxin receptor instead of an anti-eotaxin antibody may be
carried out analogously.
25

The present invention provides a process for the production of
an eotaxin, which comprises obtaining bronchoalveolar lavage
fluid obtained from a human or non-human animal challenged
with a provoking stimulus, for example, from a human suffering
30 from allergic or non-allergic asthma, or other lung disease,
or a guinea-pig sensitised with a foreign protein, and
isolating a fraction showing eosinophil chemoattractant
activity. One method of isolating an eotaxin-containing
fraction of bronchoalveolar lavage fluid is sequential cation
35 exchange, size exclusion and reversed phase HPLC systems.
The desired fraction generally contains a polypeptide having a
molecular weight in the range from 6-16kDa. Purity may be

verified by SDS-PAGE. If desired, the authenticity of the
substance obtained may be determined by comparison of the
amino acid sequence thereof with the amino acid sequence set
out in SEQ.ID. NO. 1 or SEQ.ID. NO. 2 (Figure 7 or Figure 8).

5

Eotaxins may be obtained according to the above procedure from
other sources, for example, from inflammatory exudate fluids,
or from in vitro cultures of macrophages, lymphocytes,
neutrophils, mast cells, airway epithelial cells, connective
10 tissue cells, vascular endothelial cells and eosinophils
themselves.

Alternatively, a full-length eotaxin, or a part (fragment) of
an eotaxin, for example, a polypeptide or peptide fragment,
15 may be produced by chemical synthesis, for example, according
to the Merryfield technique. A further method for producing a
full-length eotaxin or a part thereof is by recombinant DNA
technology. All methods of producing eotaxin are part of the
present invention.

20

To produce a full-length eotaxin polypeptide or a polypeptide
(or peptide) fragment by recombinant DNA technology, a nucleic
acid sequence encoding the polypeptide is inserted into an
expression vector under the control of appropriate control
25 sequences. A recombinant polypeptide may then be expressed
using a prokaryotic expression system, for example, in E.
coli, or using a eukaryotic cell system, in which case the
resulting polypeptide may be glycosylated. Such techniques
are standard see, for example Sambrook, J., Fritisch, E.F. and
30 Maniatis T., Molecular Cloning A Laboratory Manual, Cold
Spring Harbor Laboratory Press, 1989 (37).

A nucleic acid encoding all or part of an eotaxin polypeptide
may be obtained by screening a library prepared from suitable
35 cells, for example, cells from allergen-challenged guinea-pig
or human lung. Screening may be carried out using a probe
comprising sequences characteristic of an eotaxin, in

particular, sequences that distinguish the eotaxin from other
related cytokines, for example, RANTES and MIP-1α. It may be
preferable to use a long probe, for example, a probe
comprising a nucleic acid sequence encoding a full-length
5 eotaxin polypeptide.

Alternatively, the polypeptide sequence of an eotaxin may be
used to design oligonucleotide primers, for example,
degenerate primers. Primers may, for example, comprise bases
10 less specific in their interaction than the naturally-
occurring bases, for example, inosine may be used. Examples
of primers are the sense sequence

        5′ TGC TGT TTC CGI GTI ACI AAC AAA   (SEQ.ID.NO. 3)
based on the amino acid sequence CCFRVTNK, and the anti-sense
15 sequence

        5′ CAT CTT GTC IGG CTT IAT TTC   (SEQ.ID. NO. 4)
based on the amino acid sequence EIKPDKM. Such primers may be
used for amplification of reverse transcribed mRNA by the
polymerase chain reaction. This provides cDNA probes for
20 screening libraries, for example, as described above, to
isolate full length eotaxin clones. Primers may include
codons chosen on the basis of known species preference.

As indicated above, derivatives of naturally occurring
25 eotaxins are also part of the present invention. Such
derivatives include polypeptides that have one or more of the
following modifications relative to a naturally-occurring
eotaxin:
    (i)   elongation or shortening at the C-terminus;
30 (ii)   elongation or shortening at the N-terminus;
    (iii)   deletion and/or insertion of internal sequences;
    (iv)   amino acid substitutions, for example at the C- and/or
              N-terminus; and
    (v)   a different pattern of glycosylation. (There is, for
35 example, a potential O-glycosylation site at residue 70.)

Such derivatives may function as agonists for structure/

activity relationship studies or as receptor antagonists.

Anti-eotaxin antibodies and anti-eotaxin receptor-antibodies,
both polyclonal and monoclonal, may be produced according to
5 standard techniques, for example, Kohler & Milstein (38). A
full-length eotaxin may be used as antigen, or it may be
preferred to use a fragment of an eotaxin in order to produce
an antibody to a specific antigenic determinant. A naturally
occurring eotaxin may used as antigen. Alternatively, a
10 recombinant or synthetic eotaxin or eotaxin polypeptide or
peptide may be used.

Figures 1 to 18 of the accompanying drawings illustrate the
present invention. The following is a brief description of
15 the Figures. A more detailed description of the Figures is
given below and in the Examples section of this specification.

Figure 1: Protocol for the generation and assay of eosinophil
chemoattractant activity in vivo.
20 Figure 2: Time course of generation of eosinophil chemo-
attractant activity in lungs of sensitized guinea-pigs after
antigen challenge.
Figures 3, 4 and 5 all relate to the purification of eotaxin
from bronchoalveolar lavage (BAL) fluids:
25   Figure 3: Final reversed phase HPLC profile showing
absorbance at 214nm and the acetonitrile gradient.
Figure 4: Measurement of ^{111}In-eosinophil chemoattractant
activity of the fractions obtained on HPLC chromatography.
Figure 5: Measurement of ^{111}In-neutrophil chemo-
30   attractant activity; lack of significant activity observed.
Figure 6: SDS-PAGE analysis of C18 reversed phase HPLC
fractions 50-56.
Figures 7 and 8: Amino acid sequences of an isolated guinea-
pig eotaxin.
35 Figure 9: Comparison of the eotaxin sequence of Figure 7
with human MCP-1, MCP-2, MCP-3 (21), guinea-pig MCP-1 (24),
human MIP-1α, MIP-1β and RANTES (18) showing conserved

residues (shaded).

Figure 10: Comparison of ^{111}In-eosinophil accumulation in vivo using guinea-pig eotaxin and the recombinant human proteins RANTES, MIP-1α and MCP-1. Figure 11 (Inset):

Inhibition of the binding of ^{125}I-RANTES to guinea-pig eosinophils in vitro induced by eotaxin and RANTES but not by a member of the C-X-C branch of chemokines, IL-8.

Figures 12, 13 and 14 illustrate the potential of blocking the response to guinea-pig eotaxin. This makes use of the fact that human RANTES, whilst competing with eotaxin for binding sites, does not activate guinea-pig eosinophils.

Figure 12: Elevation of intracellular calcium levels in human eosinophils in vitro induced by eotaxin, RANTES and, at high concentration only, MCP-1.

Figure 13: Elevation of intracellular calcium levels in guinea-pig eosinophils in vitro induced by eotaxin, but not human RANTES and MCP-1.

Figure 14: Representative traces showing inhibition of guinea-pig eotaxin-induced elevation of intracellular calcium levels in guinea-pig eosinophils in vitro by the previous exposure of the same cells to human RANTES. Inset: Figure 15: Values (mean±SEM) for four separate eosinophil preparations.

Figure 16: Dose response curve showing inhibition of guinea-pig eotaxin-induced elevation of intracellular calcium levels in guinea-pig eosinophils in vitro by the previous exposure of the same cells to human RANTES.

Figure 17: Histogram showing comparison of in vivo response of guinea-pig eosinophils to eotaxin, RANTES and RANTES plus eotaxin co-injected intradermally.

Figure 18: Histogram showing number of eosinophils and neutrophils in bronchoalveolar lavage fluid (BAL) after administration of guinea-pig eotaxin as an aerosol to guinea-pig airways.

As in allergic asthmatic patients, exposure of sensitised guinea-pigs to aerosolised antigen results in an immediate

phase of bronchoconstriction with associated mast cell
degranulation followed, in some individuals, by a late phase
of bronchoconstriction and airway hyper-responsiveness (4-7).
Although clearly no one model mimics all the features of the
5 human disease, the guinea-pig model shares common features
with the asthmatic response in man and has been extensively
used to investigate possible mechanisms (7). In particular,
in both guinea-pig and man, the immediate response to allergen
triggers the subsequent accumulation in the lung of high
10 numbers of eosinophils. Experiments were designed to detect
the appearance in the lung of chemoattractants that may be
responsible for the accumulation of eosinophils. A strategy
was employed that has previously been applied to the
identification of neutrophil chemoattractants in inflammatory
15 exudates (8-10).

Guinea-pigs were sensitised with intraperitoneal ovalbumin on
day 1 followed by a short exposure of their lungs to
aerosolised ovalbumin at day 8. On day 15-21 animals were
20 challenged with aerosolised ovalbumin and killed at different
intervals using a barbiturate overdose. Immediately after
death the airways were lavaged with saline. The broncho-
alveolar lavage (BAL) fluid was centrifuged to remove cells,
and supernatants were either stored at -20°C for assay or
25 subjected to purification. Eosinophil chemoattractant
activity in BAL fluid samples or HPLC fractions was tested by
injecting them intradermally into assay guinea-pigs previously
given intravenous injections of ^{111}In-eosinophils (11,12).
After a 2 or 4h interval, assay animals were killed and the
30 punched out skin sites were counted in a gamma-counter (Figure
1).

Figure 2 shows the time-course of appearance of eosinophil
chemoattractant activity in BAL fluid. Significant activity
35 was observed 30min after antigen challenge. Activity
increased up to 3h, remained high at 6h, but was not
significant in 24h samples. Control samples (BAL fluid from

sham-sensitised/challenged or sensitised/sham-challenged
guinea-pigs) taken at 3h had no significant activity.

Eosinophil chemoattractant activity, which we termed eotaxin,
5 was purified from 3h BAL fluid by sequential cation exchange,
size exclusion and reversed phase HPLC using the in vivo
^{111}In-eosinophil accumulation assay to measure the activity of
fractions throughout. Eotaxin eluted as a single discreet
peak of bioactivity from both the cation exchange and the
10 size exclusion steps, indicating a strongly cationic protein
of 7-14kDa. Reversed phase chromatography separated eosino-
phil chemoattractant activity into two peaks (fractions 51+52
and fraction 54), which were associated with discreet peaks of
protein absorbance (Figures 3 and 4). Selectivity for
15 eosinophils was shown by the lack of significant neutrophil
chemoattractant activity in these fractions as measured by the
accumulation of ^{111}In-neutrophils in the skin assay (Figure
5). Histological examination of skin injected with eotaxin
(2pmol) demonstrated eosinophil accumulation at 4 and 24h,
20 particularly around small blood vessels (haematoxylin and
eosin stains).

SDS-PAGE analysis revealed a single protein band in each of
fractions 51, 52 and 54 (Figure 6). The protein in fractions
25 51 and 52 was slightly larger than that in fraction 54. This
was confirmed by mass analysis in which the major signals were
at approximately 8.81 and 8.38 kDa respectively (see Example
3). N-terminal sequencing of fractions 51, 52 and 54 revealed
identical amino acid sequences (see Figure 7). The N-terminal
30 37 residue sequence of eotaxin as set out in SEQ.ID. NO. 1 and
Figure 7 shows 57% homology with human monocyte chemotactic
protein (MCP-1 (13), otherwise known as MCAF (14) and JE
(15)). Tryptic peptides of fraction 54 were also sequenced
and readily aligned by comparison with human MCP-1 to give the
35 virtually complete sequence of eotaxin with an overall
homology of 53%, see Figure 7. It is likely that the
variations in molecular mass reflect differential

glycosylation as the four mass signals obtained (two major and
two minor, see Example 3) are all different from each other by
multiples of approximately 220 mass units. The sequence
contains no N-glycosylation sites, but a potential O-glyco-
5 sylation site at position 70 has been identified (see Example
3). Human MCP-1 also exhibits heterogeneity on SDS-PAGE due
to differences in the O-linked carbohydrate modification (16).

The platelet factor 4 superfamily of chemotactic cytokines, or
10 chemokines, is characterised by four conserved cysteines. The
relative position of the two N-terminal cysteines allows the
subdivision of this superfamily into the C-X-C chemokines (eg.
IL-8 (17)) which are predominantly neutrophil chemoattractants
and the C-C chemokines (eg. MCP-1, RANTES, MIP-1α and MIP-1β
15 (18)) which are chemotactic for leukocytes other than
neutrophils. Eotaxin is a member of the C-C branch of chemo-
kines. The greatest homology (53%) is with human MCP-1 which,
in the limited in vitro studies to date, has been reported to
be inactive on human eosinophils (19,20) and with the recently
20 described human MCP-2 (54%) and MCP-3 (51% homology calculated
on the basis of SEQ.ID. No. 1 (Figure 7); 54% homology when
calculated on the basis of the overlapping sequences) (21).
Homology with other human C-C chemokines (Figure 9) is: MIP-
1β (37% calculated on the basis of SEQ.ID. No. 1 (Figure 7);
25 39% when calculated on the basis of the overlapping
sequences), MIP-1α (31% calculated on the basis of SEQ.ID. No.
1 (Figure 7); 32% when calculated on the basis of the over-
lapping sequences) and RANTES (26% calculated on the basis of
SEQ.ID. No. 1 (Figure 7); 27% when calculated on the basis of
30 the overlapping sequences). The latter two proteins have
recently been shown to be potent eosinophil activators in
vitro (20,22) whereas MIP-1β activates lymphocytes in vitro
(23) but apparently not eosinophils (20). Eotaxin shows the
greatest structural homology with human MCP-1, MCP-2 and MCP-
35 3. Eotaxin has functional similarities, but relatively low
homology, when compared with RANTES and MIP-1α. Eotaxin is
clearly a distinct molecule from guinea-pig MCP-1; the latter

has recently been cloned (24) and it has only a 43% homology
with the eotaxin sequence, see Figure 9. Guinea-pig MCP-1 was
shown to be chemotactic for monocytes but was not tested on
eosinophils (24). Interestingly, eotaxin has a 41% homology
5 with a C-C protein whose gene is expressed in mouse mast cells
and upregulated 2h after the interaction between IgE and
antigen (25). No functional activity has been reported for
this protein but it is distinct (51% homology) from mouse MCP-
1/JE (25).
10
Guinea-pig eotaxin was very potent in inducing eosinophil
accumulation in vivo: 1-2pmol/skin site giving a 730±140%
response (mean ± s.e.m., n=18 animals) compared with saline-
injected sites. Further, marked eosinophil accumulation was
15 seen within 30 minutes of intradermal injection (Figure 10).
This is supported by experiments in vitro. The effect of
eotaxin on eosinophils in vitro was shown by (i) inhibition of
binding of ^{125}I-RANTES to guinea-pig cells (Figure 11),
(ii) elevation of cytoplasmic calcium in human and guinea-pig
20 cells (Figures 12 and 13) and (iii) chemotaxis of human
eosinophils in a Boyden chamber system: the chemotactic
responses to eotaxin and RANTES were of similar magnitude over
the range 0.1-3.0nM. In contrast to eotaxin, human
recombinant MCP-1 and RANTES did not induce guinea-pig
25 eosinophil responses in vivo or in vitro (Figures 10 and 13).
This may reflect a species difference although RANTES did
bind to guinea-pig eosinophils without inducing activation
(Figure 11, inset in Figure 10). Eotaxin has a similar
potency to RANTES on human eosinophils whereas MCP-1 is either
30 inactive (20) or active only at high doses (Figure 12). Thus,
eotaxin has a potent direct effect on both human and guinea-
pig eosinophils.

Responses of guinea-pig eosinophils to guinea-pig eotaxin in
35 vitro and in vivo can be inhibited by human RANTES. Figures
14, 15 and 16 show the response of FURA-2-loaded guinea-pig
eosinophils to guinea-pig eotaxin before and after the

addition of human RANTES. The eotaxin−induced increase in
intracellular calcium concentration is reduced to a
substantial degree when the cells are first exposed to human
RANTES, which itself fails to induce a response. Figure 17
5 shows that human RANTES, when coinjected with the eotaxin,
reduces the accumulation of guinea−pig eosinophils induced by
eotaxin in guinea−pig skin in vivo. The results support our
observations that eotaxin exhibits competitive binding with
radiolabelled human RANTES on guinea−pig eosinophils and that
10 eotaxin is a potent functional stimulant whereas RANTES is
not. Accordingly, RANTES appears to act as a receptor
antagonist for eotaxin on guinea−pig eosinophils.

Figure 18 shows that eosinophils accumulate in guinea−pig
15 airways in vivo within 24 hours of the administration of
aerosolized guinea−pig eotaxin, whereas substantially no
accumulation of neutrophils is observed.

The following Examples illustrate the present invention.
20

EXAMPLE 1
Production and testing of bronchoalveolar lavage fluid

METHODS: Male Dunkin Hartley guinea−pigs (300−400g) were
25 sensitised with intraperitoneal ovalbumin (OA, 1mg) on day 1
followed by exposure to aerosolised antigen (2% OA for 5min
using an ultrasonic nebuliser) on day 8 (6). On day 15−21,
animals were pretreated with an antihistamine to prevent acute
fatality (pyrilamine, 10mg kg^{-1}, i.p.) and challenged by
30 exposure to aerosolised antigen (1% OA for 20min). At
different times after antigen challenge animals were treated
with atropine (0.06mg kg^{-1}, i.p.) to prevent broncho−
constriction and killed with a barbiturate overdose. Broncho−
alveolar lavage was performed with 4ml saline. Samples were
35 centrifuged to remove cells and the supernatant stored at
−20°C prior to assay. BAL samples were assayed by intradermal
injection (0.1ml) into guinea−pigs previously given

intravenous injections of 5×10^6 ^{111}In-eosinophils (elicited in
donor animals by repeated intraperitoneal injections of horse
serum and purified on discontinuous Percoll gradients, >94%
purity) (11,12). After 4 hours, assay animals were killed
5 and the skin sites punched out for gamma-counting. Data are
presented as the mean ± s.e.m. and were tested by one way
analysis of variance. A p value of <0.05 was considered
statistically significant.
RESULTS: Figure 1 shows, schematically, procedures for the
10 generation and measurement of eosinophil chemoattractant
activity in vivo. Figure 2 illustrates the time course of
generation of eosinophil chemoattractant activity in lungs of
sensitised guinea-pigs after antigen challenge (filled
squares, n=4-10). Activity was measured in an in vivo skin
15 assay of ^{111}In-eosinophil accumulation in unsensitised guinea-
pigs (3 test animals per BAL sample). Significant activity
was seen in bronchoalveolar lavage samples taken 0.5, 1.5, 3
and 6h after antigen challenge (compared to responses to
intradermal saline, shown as the dotted line). No
20 significant activity was observed in 24h samples. No activity
was seen in lavage samples obtained 3h after sham (saline)
challenge of sensitised animals (filled circles, n=5) or
antigen challenge of sham (saline) sensitised animals (filled
diamonds, n=5).

25

EXAMPLE 2
Purification of eotaxin from bronchoalveolar lavage fluids

METHODS: Bronchoalveolar lavage (BAL) fluid collected from 25
30 sensitised guinea-pigs (each lavaged with 4ml, followed by
2x10ml, saline) 3h after antigen challenge (1% OA, 5min
exposure) was applied to a cation exchange HPLC column
(Ultropac TSK535CM, 7.5x150mm). Activity was eluted at approx
1.4M ammonium acetate, pH5.5, and lyophilised for size
35 exclusion HPLC (Ultropac TSK columns SWP, 7.5x75mm, G4000SW,
7.5x600mm, and G2000SW, 7.5x600mm, in series, equilibrated in
0.08% TFA). Activity eluted at approx 7-14 kDa. This was

applied to a wide pore (300A) Vydac C18 reversed phase HPLC
column (4x250mm) in 0.08% TFA, eluted with a linear gradient
of acetonitrile (0-80% ACN in 0.08% TFA, over 80mins, at 1ml
min^{-1}) and 0.5min fractions were collected. Aliquots (4%) of
5 each fraction were lyophilised in the presence of carrier
protein (BSA, <0.1ng endotoxin mg^{-1}) and redissolved in
0.8ml saline for testing in the skin bioassays of ^{111}In-
eosinophil and ^{111}In-neutrophil accumulation over 2 hours
(n=4). Eosinophils (99%, 0.5% neutrophils) were elicited with
10 repeated intra-peritoneal injections of horse serum (11,12)
and neutrophils (99.4%, 0.6% eosinophils) were elicited with a
single intraperitoneal injection of casein (5% w/v, 15ml) 17h
prior to purification on discontinuous Percoll gradients and
labelling with ^{111}In.
15

RESULTS: The results are presented in Figures 3, 4 and 5.
Figure 3 shows the final reversed phase HPLC profile showing
absorbance at 214nm and the acetonitrile gradient. Figure 4
shows that eosinophil chemoattractant activity was seen in 2
20 peaks, corresponding to fractions 51+52 and fraction 54, which
corresponded to discreet peaks of absorbance. Figure 5 shows
that no significant neutrophil chemoattractant activity was
detected in these fractions. In contrast, guinea-pig C5a des
Arg (30% zymosan-activated plasma (11), approx 10pmol/site)
25 induced the accumulation of both ^{111}In-eosinophils (5211±893)
and ^{111}In-neutrophils (9872±473). Fractions 50, 53, 55 and 56
consistently gave little or no activity in the guinea-pig skin
bioassays of leukocyte accumulation. No significant protein
absorbance was detected in the remainder of the gradient (up
30 to 80% acetonitrile).

Example 3
Purity, mass analysis and protein sequence of eotaxin

35 METHODS: 2% aliquots of each fraction were lyophilised,
redissolved in 10µl SDS buffer, heated (95°C, 5 min) and 0.3µl
run on 8-25% gradient gels in a Pharmacia Phast System. Gels

were visualised with silver staining. Mass analysis was
performed on fractions 51, 52 and 54 using a Finnigan MAT
Lasermat with α-cyano-4-hydroxycynnamic acid and sinapinic
acid matrices. Mass measurements were calibrated internally

5 using protein standards. 5% aliquots of each bioactive
fraction (51, 52 and 54) were applied directly to automated N-
terminal sequence analysis using fast cycles on an Applied
Biosystems 477A containing a microcartridge essentially as
described (32). The amino-terminal 37, 35 and 29 residues

10 were obtained for fractions 51, 52 and 54 respectively. No
differences between corresponding positions were found. The
apparent initial yields of these three analyses were all
approximately 7-8pmol. Thus fractions 51, 52 and 54 contained
approx 200pmol each, assuming 70-80% sequencing yields. Gaps

15 were found at positions 8,9 and 33, consistent with the
presence of cysteine residues at these positions.
Approximately 30pmol of fraction 54 was reduced and alkylated
by sequential treatment with 1mM dithiothreitol for 5min at
50°C and then 10mM acrylamide for 30min at 37°C before

20 digestion with alkylated trypsin (Promega) in 20mM Tris/HCl,
pH8.8, containing 0.5% Thesit. Peptides were separated using
a Reliasil C18 (300Å, 5μm) column (1x150mm) developed with a
linear acetonitrile concentration gradient in 0.08% tri-
fluoroacetic acid at 50μl/min on a Microm HPLC system.

25 Purified peptides were subjected to N-terminal sequence
analysis as above, but all four cysteine residues were
positively identified as the PTH-cys-S-ß-propionamide
derivative (33). Position 70 gave no PTH derivative in
peptides T6 and T7 and is a probable position of O-glyco-

30 sylation.

RESULTS: The results are presented in Figures 6, 7, 8 and 9.
Figure 6 is a photograph of the SDS-PAGE gel. For reference,
human IL-8 (72 amino acids, approx 8kDa) was run in lanes A, B

35 and C (12, 2.4 and 0.5ng/0.3μl/lane respectively). Laser
desorption time of flight mass analysis gave signals at
8.81kDa (major) and 9.03kDa (minor) for each of fractions 51

and 52. Fraction 54 gave signals at 8.38kDa (major) and
8.15kDa (minor).

Figures 7 and SEQ.ID. NO. 1 show the amino acid sequence of
eotaxin, which was determined by sequencing the intact

5 molecule as well as peptides derived from digestion with
trypsin (T). N-terminal analyses showed the highest homology
with human MCP-1 (57%) and the tryptic peptides were readily
aligned by comparison with the human MCP-1 sequence. Figure 8
shows the amino acids sequence as confirmed by nucleic acid

10 sequencing. Figure 9 is a comparison of the eotaxin sequence
with human MCP-1, MCP-2, MCP-3 (21), guinea-pig MCP-1 (24),
human MIP-1α, MIP-1β and RANTES (18) showing conserved
residues (shaded).

15 Example 4

In vitro and in vivo testing of eotaxin

METHODS:

(i) Eotaxin was a pool of both peaks of bioactivity

20 (fractions 51+52 and fraction 54) from the final reversed
phase HPLC separation described in Example 2 (see Figures 3, 4
and 5). ^{111}In-eosinophil accumulation in guinea-pig skin was
measured over 4h as described in Example 1 (see Figures 1 and
2). In the same animals (n=4) additional sites were injected

25 with eotaxin 30 minutes before killing.

(ii) For the binding studies, 4×10^5 eosinophils were
incubated with 0.1nM ^{125}I-RANTES and various concentrations of
cold ligand (50μl at 0°C for 2h). The Hank's buffered salt
solution contained 30mM HEPES, 10mM EDTA, 0.1% sodium azide

30 and 1% BSA at pH7.5. Results are the mean of two assays each
done in triplicate.

(iii) For measurement of intracellular calcium levels human
and guinea-pig eosinophils (10^7cells/ml in Ca^{2+}/Mg^{2+}-free PBS
+ 0.1% BSA) were loaded with fura-2-acetoxymethyl ester

35 (2.5μM, 30min at 37°C).   After two washes cells were resus-
pended at 10^6cells/ml in Ca^{2+}/Mg^{2+}-free PBS containing 10mM
HEPES, 0.25% BSA and 10mM glucose (pH7.4). Aliquots were

dispensed into quartz cuvettes and the external $[Ca^{2+}]$
adjusted to 1mM with $CaCl_2$. Changes in fluorescence were
monitored at 37°C using a Perkin Elmer LS50 spectrophotometer
at excitation wavelengths 340nm and 380nm and emission
5   wavelength 510nm. $[Ca^{2+}]_i$ levels were calculated as described
previously (34) using the ratio of the two fluorescence
readings and a Kd for Ca^{2+} binding at 37°C of 224nM. Peri-
pheral human eosinophils were prepared as described previously
(35) by density centrifugation on Percoll followed by
10 immunomagnetic removal of $CD16^+$ neutrophils using the MACS
system. Guinea-pig eosinophils were prepared as described in
Example 1 (11,12).

(iv) To test for suppression of eosinophil accumulation in
vivo using human RANTES, accumulation of [111]-labelled guinea-
15 pig eosinophils in skin sites was measured as described above.
Guinea-pigs were injected with 1.8pmol eotaxin (n=2), 100pmol
RANTES (n=1) or both 1.8pmol eotaxin and 100pmol RANTES (n=2).
Intradermal saline was used as control.

(v)   For testing of receptor antagonist activity, the fura-2-
20 acetoxymethyl ester-loaded guinea-pig eosinophils were
stimulated with 3nM eotaxin with or without pretreatment with
human RANTES.

(vi) To investigate the effect of aerosol exposure of guinea-
pigs to eotaxin, naive guinea-pigs were exposed to an aerosol
25 of either eotaxin or a control medium (n=8 per group).
Exposure was performed by placing two animals in a single
chamber and nebulising 24pmol of eotaxin dissolved in 10ml PBS
containing BSA carrier protein at 80µg/ml over a period of 35-
40 minutes. Control animals received aerosolised PBS/BSA in
30 the same manner. The animals were killed 20 hours after
exposure to eotaxin or control medium. BAL fluid (5x10ml
HBSS, 10mM EDTA, pH 7.35) was recovered and centrifuged (300g,
20min, 4°C). Total BAL cell count was determined by haemo-
cytometer and differential cell counts performed on stained
35 (DiffQuick) cytospin preparations (3 per animal, 400 cell
counts per slide).

RESULTS: The results are presented in Figures 10, 11, 12,

13, 14, 15, 16, 17 and 18.

Figure 10 shows that guinea-pig eotaxin (1.6pmol) induces significant ^{111}In-eosinophil accumulation in vivo 30min (open squares, $p<0.01$) and 4h (filled squares, $p<0.01$) after

5 intradermal injection. In contrast, the recombinant human proteins, RANTES, MIP-1α and MCP-1, at doses up to 100pmol, were without effect over 4 hours. Figure 11 inset shows that eotaxin and RANTES, but not the C-X-C chemokine IL-8, inhibit the binding of ^{125}I-RANTES ($B_0=14.4\%$) to guinea-pig

10 eosinophils in vitro. Figure 17 shows (a) that human RANTES does not induce significant eosinophil accumulation and (b) that human RANTES inhibits to a substantial degree the eosinophil accumulation induced by eotaxin, which suggests that RANTES acts as a receptor antagonist for eotaxin in vivo.

15

Figure 12 shows that eotaxin, RANTES and, at high concentration only, MCP-1 induce elevation of intracellular calcium levels in human eosinophils in vitro. Traces are with eosinophils from one donor. In two other donors 2nM eotaxin

20 gave a mean calcium elevation of 61nM. In the three donors (97.3±1.2% eosinophils) responses to 10nM RANTES were 194±74nM $[Ca^{2+}]_i$ and responses to 100nM MCP-1 were 93±38nM $[Ca^{2+}]_i$. Figure 13 shows that guinea-pig eotaxin, but not human RANTES or MCP-1, elevates intracellular calcium levels in guinea-pig

25 eosinophils in vitro. Traces are with cells from one donor. In three donors (97.5±0.8% eosinophils) responses were: 2nM eotaxin, 90±13nM $[Ca^{2+}]_i$; 100nM RANTES, 2.0±1.7nM $[Ca^{2+}]_i$; 100nM MCP-1, 3.3±0.7nM $[Ca^{2+}]_i$.

30 Figure 14 and the inset Figure 15 show that prior treatment of guinea-pig eosinophils with RANTES (100nM) inhibits the increase in intracellular free calcium levels seen when eosinophils are treated with eotaxin (3nM) alone. RANTES appears to be acting as a receptor antagonist in guinea-pig

35 eosinophils. Figure 16 is a dose-response curve using 3nM eotaxin and increasing amounts of RANTES.

Figure 18 shows that guinea-pig eotaxin, administered as an
aerosol, induces eosinophil accumulation in guinea-pig airways
in vivo, whereas substantially no accumulation of neutrophils
is observed.

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- 30 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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    (ii) TITLE OF INVENTION: CHEMOTACTIC CYTOKINE

    (iii) NUMBER OF SEQUENCES: 4

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

    (vi) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: GB 9318984
        (B) FILING DATE: 14-SEP-1993

    (vi) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: GB 9408602
        (B) FILING DATE: 29-APR-1994

(2) INFORMATION FOR SEQ ID NO: 1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 73 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (iii) HYPOTHETICAL: NO

    (vi) ORIGINAL SOURCE:
        (A) ORGANISM: Cavia porcellus
        (D) DEVELOPMENTAL STAGE: Adult
        (F) TISSUE TYPE: Bronchoalveolar lavage fluid

SUBSTITUTE SHEET (RULE 26)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

His Pro Gly Ile Pro Ser Ala Cys Cys Phe Arg Val Thr Asn Lys Lys
1   5   10   15

Ile Ser Phe Gln Arg Leu Lys Ser Tyr Lys Ile Ile Thr Ser Ser Lys
            20   25   30

Cys Pro Gln Thr Ala Ile Val Phe Glu Ile Lys Pro Asp Lys Met Ile
            35   40   45

Cys Ala Asp Pro Lys Xaa Xaa Trp Val Gln Asp Ala Lys Lys Tyr Leu
    50   55   60

Asp Gln Ile Ser Gln Xaa Thr Lys Pro
65   70

(2) INFORMATION FOR SEQ ID NO: 2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 73 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: protein

  (iii) HYPOTHETICAL: NO

   (vi) ORIGINAL SOURCE:
        (A) ORGANISM: Cavia cobaya
        (F) TISSUE TYPE: Bronchial lavage fluid

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

His Pro Gly Ile Pro Ser Ala Cys Cys Phe Arg Val Thr Asn Lys Lys
1   5   10   15

Ile Ser Phe Gln Arg Leu Lys Ser Tyr Lys Ile Ile Thr Ser Ser Lys
            20   25   30

Cys Pro Gln Thr Ala Ile Val Phe Glu Ile Lys Pro Asp Lys Met Ile
            35   40   45

Cys Ala Asp Pro Lys Lys Lys Trp Val Gln Asp Ala Lys Lys Tyr Leu
    50   55   60

Asp Gln Ile Ser Gln Thr Thr Lys Pro
65   70

(2) INFORMATION FOR SEQ ID NO: 3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

- 32 -

        (ii) MOLECULE TYPE: DNA (primer)

       (iii) HYPOTHETICAL: YES

       (iii) ANTI-SENSE: NO

        (ix) FEATURE:
             (A) NAME/KEY: modified_base: N is inosine
             (B) LOCATION: 12.

        (ix) FEATURE:
             (A) NAME/KEY: modified_base: N is inosine
             (B) LOCATION: 15

        (ix) FEATURE:
             (A) NAME/KEY: modified_base: N is inosine
             (B) LOCATION: 18

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TGCTGTTTCC GNGTNACNAA CAAA   24

(2) INFORMATION FOR SEQ ID NO: 4:

         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 21 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (primer)

       (iii) HYPOTHETICAL: YES

       (iii) ANTI-SENSE: YES

        (ix) FEATURE:
             (A) NAME/KEY: modified_base: N is inosine
             (B) LOCATION: 10

        (ix) FEATURE:
             (A) NAME/KEY: modified_base: N is inosine
             (B) LOCATION: 16

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CATCTTGTCN GGCTTNATTT C   21